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Biological Modification of Trichothecene Mycotoxins: Acetylation and Deacetylation of Deoxynivalenols by *Fusarium* spp.

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Attempts were made to elucidate the acetyl transformation of novel trichothecene mycotoxins, 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (deoxynivalenol) and its derivatives, by trichothecene-producing strains of *Fusarium nivale*, *F. roseum*, and *F. solani*. In the peptone-supplemented Czapek-Dox medium, *F. roseum* converted 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-acetyldeoxynivalenol) to deoxynivalenol. 3-Acetyldeoxynivalenol was also deacetylated by intact mycelia of the three strains in sugar-free Czapek-Dox medium. The growing *F. nivale* acetylated deoxynivalenol to afford a small amount of 3-acetyldeoxynivalenol. 3α,7α,15-Triacetoxy-12,13-epoxytrichothec-9-en-8-one (deoxynivalenol triacetate) was transformed by the intact mycelium of *F. solani* into 7α,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-en-8-one (7,15-diacetyl-deoxynivalenol), which was then deacetylated to give 7α-acetoxy-3α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (7-acetyldeoxynivalenol). It was noted that the ester at C-7 was not hydrolyzed by the fungal mycelium.

Within the past several years, a group of structurally related compounds, called trichothecenes, has been isolated from several different species of toxic fungi: *Trichothecium*, *Cephalosporium*, *Myrothecium*, *Fusarium*, and *Trichoderma*. The individual metabolite showed the evident difference in the modification of a tetracyclic 12,13-epoxytrichothec-9-ene nucleus, such as oxidation of some carbon atoms to afford ketone or alcohol, and esterification of the resultant alcohol. It was suggested that these structural differences affect the selectivity and specificity of biological activity, including mammalian toxicity, antibiotic activity, insecticidal activity, cytotoxicity, and phytotoxicity (1, 3).

However, very little is known concerning the biological transformation of trichothecenes and its significance in biological activity. Horvath and Varga (4) reported evidence that the isocrotonic ester group of trichothecin and crotocin was enzymatically hydrolyzed by *Penicillium chrysogenum* Thom. Recently, Ellison and Kotsonis (2) reported that incubation of T-2 toxin with supernatant fractions of both human and bovine liver homogenates resulted in the conversion to HT-2 toxin.

In the present paper, the authors attempted to elucidate the mode of microbial transformation of the novel trichothecenes, deoxynivalenol and its derivatives (12), by the trichothecene-producing strains of *Fusarium nivale*, *F. roseum*, and *F. solani*.

MATERIALS AND METHODS

Microorganisms and chemicals. Stock cultures of *F. nivale* Fn-2B, *F. solani*, and *F. roseum* (no. 117) were maintained on Czapek-Dox agar. They were stored at 4 C and subcultured bimonthly. These fungal species were previously demonstrated to metabolize the following trichothecene mycotoxins: *F. nivale*, fusarenon (6, 7, 9, 10); *F. solani*, neosolaniol and T-2 toxin (8); *F. roseum*, deoxynivalenol and its monoacetate (5, 12).

Analytically pure 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (deoxynivalenol, compound I) and 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-acetyldeoxynivalenol, compound II) were isolated from culture broth of *F. roseum* as previously described (5, 12). 3α,7α,15-Triacetoxy-12,13-epoxytrichothec-9-en-8-one (deoxynivalenol triacetate, compound III) was prepared from deoxynivalenol by acetylating with acetic anhydride-pyridine (12). Other chemicals were reagent grade.

Trichothecene conversion by growing cells. Growing mycelia of fungus that had been grown on peptone-supplemented Czapek-Dox medium (pH 6.8) in a shaking flask at 20 C for 3 days were washed three times with 0.67 M phosphate buffer (pH 6.8). The wet

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mycelium (2 g) was resuspended in 50 ml of Czapek-Dox medium containing 0.02% trichothecene as the sole carbon source. Incubation was performed at 25 C with shaking.

Trichothecene determination. Physicochemical properties of trichothecenes were determined with the following apparatus: melting point, Yanagimoto melting point apparatus (model MP-S2); infrared spectrum, Hitachi model EPI-G2 double-beam infrared spectrophotometer; ultraviolet absorption spectrum, Hitachi model 124 recording spectrophotometer; proton magnetic resonance spectrum, Hitachi model R-22 high resolution nuclear magnetic resonance spectrometer; mass spectrum, JEOL model JMS-O7 mass spectrometer; thin-layer chromatography (TLC), Kieselgel GF_{254} (E. Merck AG); gas-liquid chromatography (GLC), Hitachi model 063 gas chromatograph equipped with hydrogen ionization detectors.

At the end of the incubation period, the mycelia was filtered off. The whole filtrate was extracted three times with equal amounts of ethyl acetate. The extract was dried over anhydrous sodium sulfate and evaporated to dryness under vaccum.

The course of trichothecene modification was followed by TLC on a silica gel plate developed in chloroform-methanol (97:3 and 5:1), chloroform-acetone (3:1 and 3:2), and ethyl acetate-toluene (3:1). The chromatoplates were viewed under a short-wave ultraviolet lamp, sprayed with 20% aqueous sulfuric acid, and heated at about 110 C for 10 min.

The transformation products were separated from the crude extract by column chromatography on silica gel using chloroform-acetone (3:2), recrystallized, and identified from their spectroscopic properties as shown in the following sections.

For quantitative estimation of trichothecenes, the crude extracts were reacted with trimethylsilylating reagent and gas chromatographed. The column was a coil (1 m by 3 mm) of stainless-steel tubing packed with 3% of OV-17 on 60- to 80-mesh Chromosorb W. The operating conditions were: column temperature, 240 C; flow rate of nitrogen, 75 ml/min; hydrogen, 0.6 kg/cm²; and air, 1.2 kg/cm². Results were expressed as percentage of total peak heights of trichothecene derivatives.

RESULTS

Transformation of compound II by F. roseum in the culture broth. To determine the time course of compound II production and the transformation of it, *F. roseum* (strain 117) was surface cultured on peptone-supplemented Czapek-Dox medium at 25 C. An entire flask containing 500 ml of the culture broth was harvested at desired intervals to estimate the concentration of trichothecenes, dry weight of the fungal mat, and pH value of the filtrate.

Maximal growth of the fungus was attained after 14 days (Fig. 1). As compound II exhibited a maximal level within 14 days of incubation

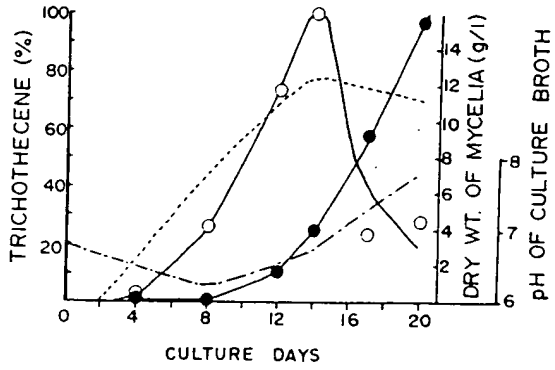
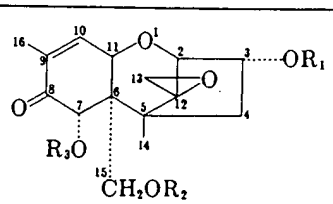


FIG. 1. *Time course of fungal growth, toxin formation, and changes in pH in the culture broth of F. roseum. The fungus was cultured on peptone-supplemented Czapek-Dox medium at 25 C. Symbols:* ○, *3-acetyldeoxynivalenol;* ●, *deoxynivalenol;* -----, *fungal growth;* — · — ·, *pH value.*

TABLE 1. *Physicochemical properties of deoxynivalenol and its derivatives*

Compound	R_1	R_2	R_3	Name	mp (C)	R_f[a]	t_R (min)[b]
I	H	H	H	3α,7α-15-trihydroxy-12,13-epoxytrichothec-9-en-8-one(deoxynivalenol)	151–153	0.09	0.9
II	Ac	H	H	3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one	185.5–186	0.40	1.6
III	Ac	Ac	Ac	3α,7α,15-triacetoxy-12,13-epoxytrichothec-9-en-8-one	155–157	0.72	4.9
IV	H	Ac	Ac	7α,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-en-8-one	147–148	0.40	2.75
V	H	Ac	H	7α-acetoxy-3α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one	194	0.09	1.6



[a] Values obtained from TLC developed in chloroform-methanol (97:3).
[b] Retention times on GLC.

followed by rapid decrease, compound I accumulated in the filtrate. The production of compound II and its disappearance were coincident with the fungal growth and the accumulation of compound I, respectively (Table 1).

Acetylation of compound I by mycelium of F. nivale. Compound I was converted with growing *F. nivale* into a compound having a higher retention time (t_R) (1.6 min) on GLC and a larger R_f value on TLC than those of the substrate (Fig. 2). From its behavior on the chromatograms, the transformation product was identified as 3-acetyldeoxynivalenol: 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (compound II). This reaction occurred within a 12-h incubation period, and the rate of transformation in the filtrate was approximately 5% after 24 h. When compound I was used as a substrate for growing mycelium of *F. roseum* or *F. solani*, little if any transformation product in the filtrate was detected on TLC and GLC.

Deacetylation of compound II by mycelia of Fusarium spp. When compound II was incubated with growing mycelium of *F. roseum*, 2% of the substrate was deacetylated after 24 h to give deoxynivalenol in the filtrate. No other product was detected on either TLC or GLC. Ten percent of compound II was also transformed into deoxynivalenol after 24 h by the mycelium of *F. nivale*. On the other hand, compound II was quantitatively converted with growing *F. solani* into deoxynivalenol within a 12-h incubation period. Although the transformation patterns of compound II by the mycelia of *Fusarium* spp. were similar, the substrate was deacetylated at an extensively higher rate by the mycelium of *F. solani* (Fig. 3).

Deacetylation product (I) was purified by repeated crystallization from ethyl acetate-petroleum ether; the mp found was 151 to 153 C. A melting point in admixture of it with the authentic sample produced no melting depression, and infrared proton and magnetic resonance spectra of the sample were identical with those of corresponding authentic standard.

Deacetylation of compound III by mycelium of F. solani. Deoxynivalenol triacetate was incubated with the mycelium of *F. solani*, and transformation products were periodically detected on TLC (solvent system; chloroform-methanol, 97:3) or on GLC as trimethylsilylated derivatives.

The triacetate (R_f, 0.72; t_R, 4.9 min) disappeared within a 6-h incubation period, and further incubation gave two transformation products, product A (R_f, 0.40; t_R of the trimethylsilylated derivative was 2.75 min) and product B (R_f, 0.09; t_R of the trimethylsilylated derivative was 1.60 min) (Fig. 4). Considering the transformation pattern of the triacetate, product B seemed to be metabolized via product A.

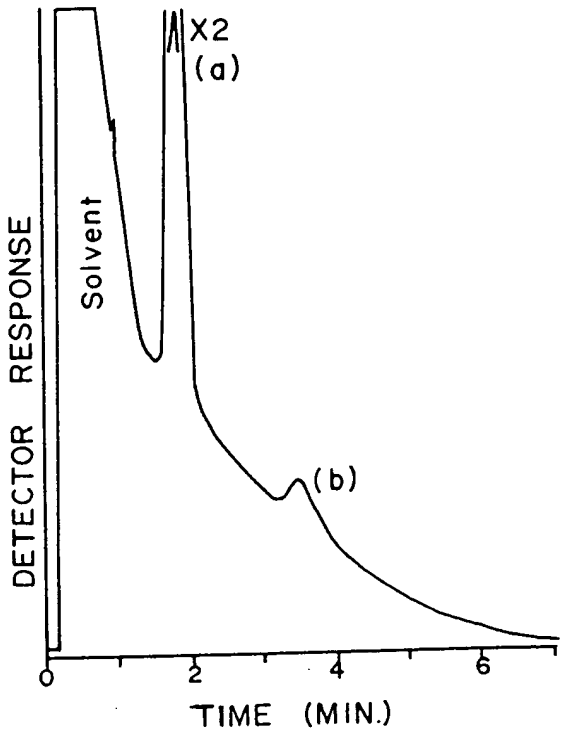


FIG. 2. *Formation of monoacetylated product (3-acetyldeoxynivalenol) from deoxynivalenol on incubation with the intact mycelium of F. nivale. The fungus was cultured on sugar-free Czapek-Dox medium. (a) Trimethylsilylated deoxynivalenol; (b) trimethylsilylated 3-acetyldeoxynivalenol.*

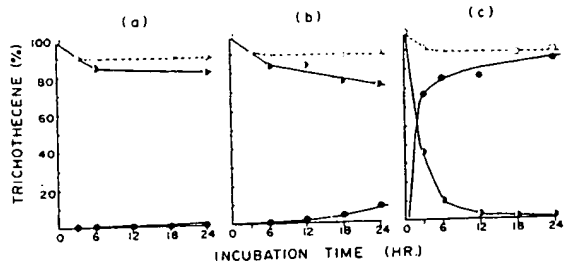


FIG. 3. *Time course of the deacetylation of 3-acetyldeoxynivalenol by intact mycelia of Fusarium spp. The fungi were cultured on sugar-free Czapek-Dox media. (a) F. roseum; (b) F. nivale; (c) F. solani. Symbols: ○, 3-acetyldeoxynivalenol of control; ◑, 3-acetyldeoxynivalenol incubated; ●, deoxynivalenol transformed.*

Isolation of products A and B from the reaction mixture. The triacetate (380 mg) was

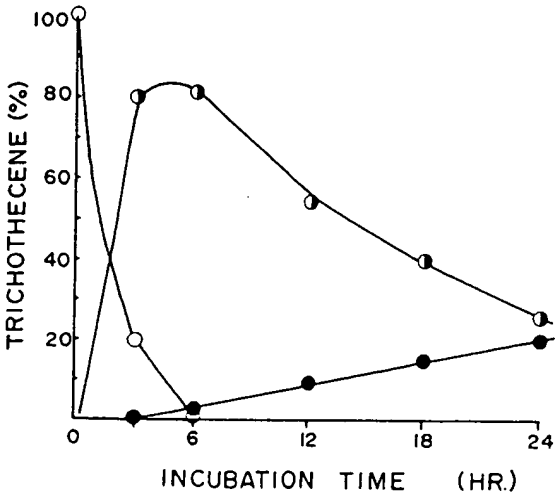


FIG. 4. *Time course of the metabolism of deoxynivalenol triacetate by the intact mycelium of F. solani. The fungus was cultured on sugar-free Czapek-Dox medium. Symbols:* ○, *deoxynivalenol triacetate;* ◑, *7,15-diacetylideoxynivalenol;* ●, *7-acetyldeoxynivalenol.*

added to 300 ml of the mycelium suspension in a 500-ml shaking flask, which was shaken at 25 C for 30 h. The mixture was filtrated by suction. The filtrate was extracted three times with a 200-ml portion of ethyl acetate. The extract was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue (370 mg) was dissolved in 2 ml of chloroform and charged on a silica gel column (1 by 25 cm) followed by elution with chloroform-acetone (3:2). The major eluate (280 mg) was recrystallized from benzene-petroleum ether to give pure product B: mp, 147 to 148 C (hexagonal plates); ultraviolet λ_{max}^{MeOH}nm: 227 (ϵ, 5,700); infrared ν_{max}^{KBr}cm^{-1}: 3,530, 1,740, 1,700, 1,660; mass spectrum (*m/e*): 380 (M^+). Analysis found: C, 59.97; H, 6.35%. Calculated for $C_{19}H_{24}O_8$: C, 59.99; H, 6.34%. Proton magnetic resonance $\delta_{Me_4Si}^{CDCl_3}$: 0.93 (3H, *s*), 1.85 (3H, *d*), 1.90 (3H, *s*), 2.20 (3H, *s*), 2.77 and 3.08 (each 1H, *d*), 3.61 (1H, *d*), 4.33 (2H, *s*), 4.52 (1H, *m*), 4.95 (1H, *d*), 6.09 (1H, *s*), and 6.61 (1H, *dd*). From these results, product B was identified as 7α,15-diacetoxy-3α-hydroxy-12,13-epoxytrichothec-9-en-8-one (compound IV).

The minor eluate (35 mg) was recrystallized from methanol to afford product A: mp 194 C (rectangular plates); infrared ν_{max}^{KBr}cm^{-1}: 3,500, 3,360, 1,730, 1,690, 1,655; mass spectrum (*m/e*): 338 M^+). Analysis found: C, 59.80; H, 6.49%. Calculated for $C_{17}H_{22}O_7$: C, 60.35; H, 6.55%. Proton magnetic resonance $\delta_{Me_4Si}^{CDCl_3}$: 1.02 (3H, *s*), 1.91 (3H, *d*), 2.27 (3H, *s*), 2.82 and 3.13 (each 1H, *d*), 3.66 (1H, *d*), 3.94 (2H, *s*), 4.56 (1H, *m*), 5.02 (1H, *d*), 6.10 (1H, *s*), and 6.64 (1H, *dd*). These results show that product A is 7α-acetoxy-3α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (7-acetyldeoxynivalenol, compound V).

DISCUSSION

Deoxynivalenol lacking a C-4 hydroxy group is a novel mycotoxin compared to the known trichothecenes, all of which have this functional group. The toxin was isolated from naturally infected barley grains with *Fusarium* spp. (5). Recently, it was also isolated from the infected corn by a Northern Regional Research Laboratory group (11). In the synthetic medium of *F. roseum*, the toxin was converted from its monoacetate (3-acetyldeoxynivalenol), accumulated by the mycelium in the phase of linear growth (Fig. 1). The monoacetate is more toxic to mice than deoxynivalenol, but the latter shows higher cytotoxicity or vomiting toxicity than the monoacetate (13). These facts led to the suggestion that deoxynivalenol found in both field crops of Japan (5) and northwest Ohio (11) was transformed from the monoacetate by biological and/or nonbiological hydrolysis during the growth and storage of the cereal grains.

By incubating deoxynivalenol and its derivatives with *F. nivale* or *F. solani*, which produces trichothecene mycotoxins having a C-4 hydroxy group, oxidation of the trichothecene nucleus, including the conversion of deoxynivalenol into nivalenol or cleavage of the ethylene oxide ring, was not detected. However, *F. nivale* gave a deoxynivalenol monoacetate by acetylation of the substrate (Fig. 2). Since the acetylation is an endogernic process, the reaction might be progressed more efficiently by adding a coenzyme such as adenosine 5'-triphosphate or acetyl coenzyme to the reaction system.

The hydrolytic deacetylation of the monoacetate by the growing mycelia proceeded readily to give deoxynivalenol, though there was an appreciable difference in the degree of reaction between three fungal strains. Among them, marked reactivity of *F. solani* was noted. No reaction occurred in the culture filtrate of *F. solani* or sugar-free Czapek-Dox solution. These results suggest the participation of intracellular enzyme in this microbial hydrolysis.

The specificity of enzymatic hydrolysis by the mycelium of *F. solani* for deoxynivalenol triacetate is given in Fig. 4. The intact mycelium hydrolyzed the C-3 ester at a faster rate than the C-15 ester, and the C-7 ester was not at all eliminated. The instability of the C-3 ester bond is also shown in Fig. 3. These results lead

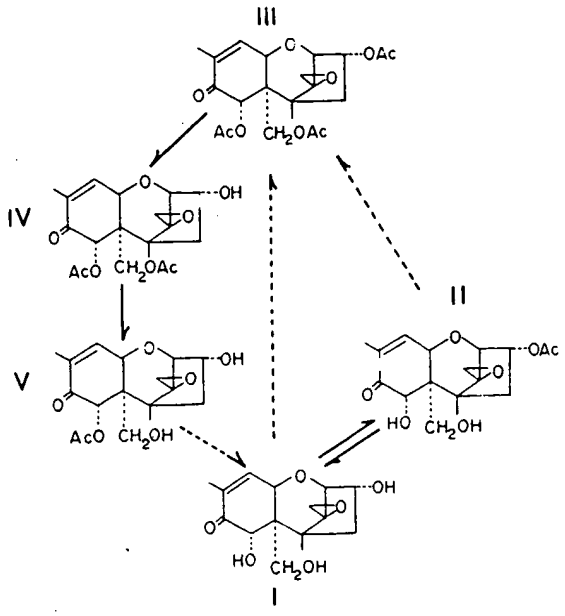


FIG. 5. *Transformation pathways of deoxynivalenols by mycelia of Fusarium spp. Symbols:* ——, *microbial transformation;* -----, *chemical transformation.*

to an assumption that the regiospecificity of C-3 ester for the enzyme is stronger than those of the other two ester bonds. From the results given above, the transformation pathway of deoxynivalenols is shown in Fig. 5. It should be noted that the ester at C-7 was not hydrolyzed by the fungal hydrolytic enzyme. Deacetylation of trichothecene mycotoxins by mammalian tissues is being investigated.

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LITERATURE CITED

1. Bamburg, J. R., and F. M. Strong. 1971. 12,13-Epoxytrichothecenes. *In* S. Kadis, A. Ciegler, and S. J. Ajl (ed.), Microbial toxins, vol. 7, Fungal toxins. Academic Press Inc., New York.
2. Ellison, B. A., and F. N. Kotsonis. 1974. In vitro metabolism of T-2 toxin. Appl. Microbiol. **27**:423-424.
3. Grove, J. F. 1969. The cytotoxicity of some transformation products of diacetoxyscirpenol. Chem. Commun. **1969**:1473-1478.
4. Horvath, I., and J. M. Varga. 1961. Enzymatic inactivation of trichothecin and crotocin. Nature (London) **192**:88.
5. Morooka, N., N. Uratsuji, T. Yoshizawa, and H. Yamamoto. 1972. Studies on the toxic substances in barley infected with *Fusarium* spp. Jap. J. Food Hyg. **13**:368-375.
6. Saito, M., and T. Tatsuno. 1971. Toxins from *Fusarium nivale*. *In* S. Kadis, A. Ciegler, and S. J. Ajl (ed.), Microbial toxins, vol. 7, Fungal toxins. Academic Press Inc., New York.
7. Tsunoda, H., N. Toyasaki, N. Morooka, N. Nakano, H. Yoshiyama, K. Okubo, and M. Isoda. 1968. Researches on the microorganisms which deteriorate the stored cereals and grain. Detection of injurious strains and properties of their toxic substance of scab fusarium blight grown on the wheat. Shokuryo Kenkyujo Kenkyu Hokoku **23**:89-116.
8. Ueno, Y., K. Ishii, K. Sakai, S. Kanaeda, H. Tsunoda, T. Tanaka, and M. Enomoto. 1972. Toxicological approaches to the metabolites of *Fusaria*. IV. Microbial examination on "bean hulls poisoning of horses" with the isolation of toxic trichothecenes, neosolaniol and T-2 toxin, of *Fusarium solani* M-1-1. Jap. J. Exp. Med. **42**:187-203.
9. Ueno, Y., K. Saito, and H. Tsunoda. 1970. Isolation of toxic principles from the culture filtrate of *Fusarium nivale*, p. 120. *In* Proc. 1st U.S.-Japan Conf. Toxic Microorganisms, Honolulu, Hawaii.
10. Ueno, Y., I. Ueno, T. Tatsuno, K. Okubo, and H. Tsunoda. 1969. Fusarenon-X, a toxic principle of *Fusarium nivale*-culture filtrate. Experientia **25**:1062.
11. Vesonder, R. F., A. Ciegler, and A. H. Jensen. 1973. Isolation of the emetic principle from *Fusarium graminearum*-infected corn. Appl. Microbiol. **26**:1008-1010.
12. Yoshizawa, T., and N. Morooka. 1973. Deoxynivalenol and its monoacetate: new trichothecene mycotoxins from moldy barley. Agric. Biol. Chem. **37**:2933-2934.
13. Yoshizawa, T., and N. Morooka. 1974. Studies on the toxic substances in the infected cereals (III). Acute toxicities of new trichothecene mycotoxins: deoxynivalenol and its monoacetate. Jap. J. Food Hyg. **15**:261-269.